



ECOTOX - BRASIL

Ecotoxicol. Environ. Contam., v. 12, n. 1, 2017, 75-84
doi: 10.5132/eec.2017.01.10

EEC

Co-exposure effects of Titanium Dioxide Nanoparticles and Metals on antioxidant systems and DNA in the fish *Hoplias intermedius*

DISNER, G.R.^{1*}; GUILOSKI, I.C.²; KLINGELFUS, T.¹; OYA SILVA, L.F.¹; LIROLA, J.R.¹;
SILVA DE ASSIS, H.C.²; CESTARI, M.M.¹

¹Environmental Mutagenesis Laboratory, Genetics Department, Federal University of Paraná, Curitiba- Brazil.

²Environmental Toxicology Laboratory, Pharmacology Department, Federal University of Paraná, Curitiba- Brazil.

(Received August 08, 2017; Accept October 10, 2017)

Abstract

Metallic nanoparticles are one of the most produced and used engineered nanomaterial and their wide applications lead to environmental contamination. The aquatic environment is the major recipient of wastes containing nanoparticles and other co-occurring contaminants. We aimed to evaluate genotoxic and biochemical effects of acute exposure to nano-TiO₂ in the fish *Hoplias intermedius* and the interaction to metals. Besides assessing the nanoparticles' physical-chemical properties we performed an acute exposure with 0.1; 1; 10 µg g⁻¹ nano-TiO₂, alone and with lead (21 µgg⁻¹) and aluminium (50 µgg⁻¹). A set of biomarkers were evaluated in the liver such as genotoxicity by comet assay and biochemical biomarkers (SOD, CAT, GPx, GSH, EROD, GST). Most of the biomarkers were altered by the metals, and the nanoparticles caused decrease in SOD (0.1 and 1 µg g⁻¹), GSH (1 µg g⁻¹), and GST (0.1 µg g⁻¹). In co-exposure, some metal effects were attenuated. There was an increase in EROD activity for most co-exposure groups. Nano-TiO₂ was not genotoxic in the experimental conditions. We did not observe any increase in DNA breaks in co-exposure, although, nanoparticles changed the response of some biochemical biomarkers.

KEY WORDS: Comet assay. Complex mixtures. Ecotoxicology. Emerging contaminants. Genotoxicity. Nano-TiO₂.

INTRODUCTION

Titanium dioxide nanoparticles (Nano-TiO₂) is one of the most produced and used engineered nanomaterial (ENM) worldwide, with an annual production in United States of about 10,000 tonnes per year (Sun *et al.*, 2014; Vance *et al.*, 2015). Nano-TiO₂ has a broad application mostly in coatings, paints, pigments, electronics, optics, cosmetics, energy, environmental applications, and as catalysts (Shaw & Handy, 2011). The extensive production increase and use of ENM will lead to environmental exposure by the generation of effluents or wastewaters, raising concern over environmental risks and impacts of nanotechnology (Clemente *et al.*, 2012). Nano-TiO₂ is the most significant ENM in terms of exposure, based on estimated release and use. Keller and colleagues (2013) estimated that 63-91% of over 260,000-309,000 metric tons of

global ENM production in 2010 ended up in landfills, released in different compartments, such as into soils, water bodies, and atmosphere.

The care about environmental risks of ENM are raising, however, currently not much is known about their concentrations in the environment. In aquatic ecosystems, nano-TiO₂ may adsorb co-occurring chemical stressors, such as Cu(II), Cr(III), Mn(II), Ni(II), Zn(II), Cd(II), Mo(VI) and alter their uptake (Kaur & Gupta, 2009; Tan & Wang, 2017). Zhang *et al.* (2007) found that carps exposed to cadmium in the presence of nano-TiO₂ accumulated 146% more Cd than controls. Nonetheless, there is little information about nano-interactions with others metals such as lead (Pb) and Aluminium (Al), considering that those two metals are commonly widespread at polluted environments.

*Corresponding author: Rodrigo Disner; e-mail: disner.rodrigo@gmail.com

Some research has shown that nanoparticles can readily penetrate a variety of cells, as their small size facilitates uptake across epithelial and endothelial cells (Kumar *et al.*, 2011; Tavares *et al.*, 2013). Evidence suggests that nanometals can cause a range of sublethal effects in fish, including respiratory toxicity, disturbances to trace elements in tissues, inhibition of Na^+K^+ -ATPase, and oxidative stress (Shaw & Handy, 2011). A study suggests that toxicity is greatly increased by harmful metals that go along with nanoparticles. A combination of metal and nanoparticle would be able to exceed the membrane barrier, and generate reactive oxygen species (ROS) within the cell, which is the most known kind of damage that nanoparticles could cause (Wang *et al.*, 2017).

Both Pb and Al are well known chemicals, applied in a wide array of commercial and industrial applications, and have been widely studied (Klingelful *et al.*, 2015; Rybak *et al.*, 2017; Saghazadeh & Rezaei, 2017; Ferraro *et al.*, 2004).

Although the findings in the scientific literature are controversial about the toxicity of nanoparticles, information over safety and potential hazards is urgently needed. In this study, we selected the freshwater fish *Hoplias intermedius* as a test organism due to its importance as a top predator in the food chain, human consumption, and wide occurrence, especially at the Rio Doce watershed, in Brazil. Within this context, the aim of the present study was to investigate the genotoxic and biochemical effects caused by nano-TiO₂ and metals (Pb and Al) co-exposure after acute treatment on a freshwater fish.

MATERIAL AND METHODS

Chemicals

The nano-TiO₂ used in this study was purchase from Sigma-Aldrich® [Titanium (IV) oxide nanopowder, 21 nm particle size (TEM), ≥99.5% trace metals basis] (Fig. 1). Nanoparticles suspensions were prepared in ultrapure water at the range of 0.01, 0.1, to 1.0 mg mL⁻¹. Immediately before exposition, the suspensions were sonicated in the ultrasonic bath (60 Hz) for 30 minutes for dispersing and to avoid aggregation. The stock solutions of lead and aluminium were prepared in ultrapure water at concentrations of 2.1 and 5.0 mg mL⁻¹, respectively. The dose of lead, 21 µg g⁻¹, applied in the present experiments was previous demonstrated to be effectively genotoxic by comet assay for both erythrocytes and kidney cells of a fish especie in the genus *Hoplias* (Ramsdorf *et al.*, 2009). The dose of aluminium was based on a previous work of Costa (2011), where this dose of aluminium increased significantly the DNA damage on erythrocytes and hepatic cells of the fish *Rhamdia quelen*.

Characterization of nano-TiO₂

Transmission Electron Microscopy (TEM) and X-Ray Diffraction technique were used for the nanoparticle

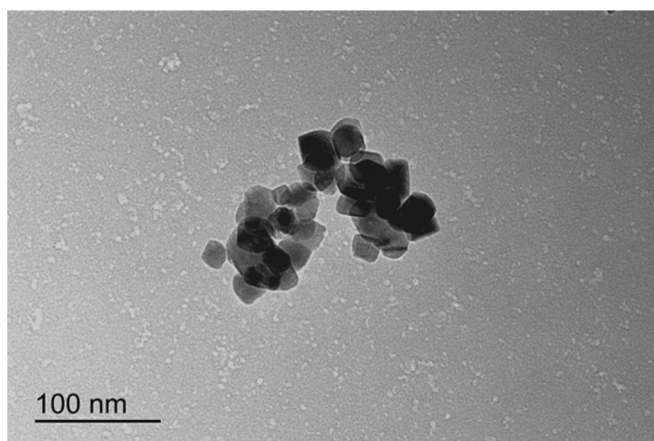


Figure 1: Titanium Dioxide Nanoparticles (Nano-TiO₂). Transmission Electron Microscopy, 300,000 X.

powder description. Zetasizer® Nano Series ZS90 (Malvern Instruments, Worcestershire, UK) was used to evaluate physical-chemical properties of the suspensions.

Experimental Design

The freshwater fish *Hoplias intermedius* were donated by the Pisciculture and Hydrobiology Station of Furnas. Once in the laboratory, they were housed in tanks (2,000 L) equipped with water filters and air pumps, and filled with dechlorinated water, for acclimatization during 2 months. They were fed everyday with supplemented commercial feed. The fish (15 per treatment) were randomly selected and distributed individually in 18 L tanks. Experiments were carried out under 27±1°C and 12:12 light-dark cycle. The average weight of the animals used in the experiment was 15.31 ± 3.99 g and size 12.86 ± 1.2 cm (mean and standard deviation). None of the fish died during the experiment. As the exposure route, we choose intraperitoneal injection as a way to guarantee the correct delivery of the dosage proposed, especially treating with nanomaterial that tend to aggregate and disperse in aqueous exposure. The treatments were: Negative control (NC); Lead (Pb) 21 µg g⁻¹; Aluminum (Al) 50 µg g⁻¹; nano-TiO₂ 0.1 µg g⁻¹ (NP1); nano-TiO₂ 1.0 µg g⁻¹ (NP2); nanoTiO₂ 10 µg g⁻¹ (NP3); and the co-exposure: Pb+NP1; Pb+NP2; Pb+NP3; Al+NP1; Al+NP2; Al+NP3. The animals received the intraperitoneal injections and 96 hours later they were anaesthetized with 150 mg L⁻¹ of benzoacaine (Gontijo *et al.*, 2003) to collect the liver samples. The animals were not fed throughout the experimental period. We performed out all the procedures in accordance with animal welfare, approved by the Ethical Committee in Animal Experimentation of Federal University of Paraná, under the protocol 977/2016.

Comet assay

The alkaline single cell gel electrophoresis test was carried out according to Singh *et al.* (1988) with modifications by Ramsdorf *et al.* (2009). A tiny piece of liver tissue (10 µg) was mechanically desegregated in 500 µL of fetal bovine serum.

Then 30 μL of the suspension were mixed with 120 μL of low melting point agarose (LMP) 0.5% (0.1g agarose, 20 mL phosphate buffered saline- PBS) and immediately placed on a slide pre-coated with normal agarose 1.5% (1.5 g, 100 mL PBS), covered with coverslip and kept refrigerated for 10 minutes. Subsequently, the coverslips were removed and the slides were placed in lysis solution (10 mM Tris ; 100 mM dimethylethylene acetic acid (EDTA); 2.5 M chloride sodium (NaCl) ; dimethyl sulfoxide (DMSO) 10%) for 24 hours. After that, the slides were kept in alkaline buffer solution [300 mM sodium hydroxide (NaOH); 1 mM EDTA] for 25 minutes to unwinding the DNA and then electrophoresis was performed for 25 minutes at 25 volts and 300 mA. The slides were neutralized with a pH 7.4 buffer and fixed with absolute ethanol for five minutes. Comet formation was observed using a LEICA® epifluorescence microscopy after ethidium bromide staining. For each fish, 100 nucleoids were counted and visually categorized according to damage, ranging from class 0 to 4 (Collins *et al.*, 1997), and a score was calculated with the sum of nucleoids number of each class multiplied by its respective class.

Biochemical analysis

Samples of liver were thawed on ice, weighed, and homogenized in potassium phosphate buffer (0.1 M pH 7.0) 1:10 (w/v), and then were centrifuged at 15,000 \times g for 30 minutes at 4°C. The supernatants were stored at -80°C until further analysis.

Superoxide dismutase (SOD)

We measured the SOD activity by analyzing the ability of SOD to inhibit the autoxidation of pyrogallol acid (Gao *et al.*, 1998), at 440 nm, in spectrophotometer. The supernatant was diluted 1:10 (v/v) in 0.1 M potassium phosphate buffer (pH 7.0). In a microtube, 885 μL of buffer (1M Tris, 5 mM EDTA, pH 8.0) and 40 μL of sample were added. After agitation, we added 50 μL of 15 mM pyrogallol and the solution was incubated for 30 minutes. The reaction was stopped with 25 μL of 1N HCl. SOD activity was expressed as U mg^{-1} of protein. We described the amount of enzyme required to cause 50% inhibition as a unit of SOD.

Catalase (CAT)

We determined the CAT activity by the Aebi method (1984), which is based on the consumption of exogenous H_2O_2 by CAT, generating water and oxygen, with a gradual decrease in absorbance at 240 nm. The supernatant was mixed with a reaction medium (295 μL ; 20 mM H_2O_2 , 50 mM Tris-base, 0.25 mM EDTA, pH 8.0) in a microplate and absorbance decrease was measured for 1 min at 27°C and the activity was expressed as $\mu\text{mol min}^{-1}.\text{mg}$ of protein⁻¹.

Glutathione Peroxidase (GPx)

We analyzed the GPx activity following the indirect method (Paglia & Valentine, 1967), from the reduction

of oxidized glutathione (GSSG) to GSH in the presence of NADPH by glutathione reductase (GR) and a decrease in absorbance at 340 nm. Volumes of 10 μL of supernatant and 130 μL of reaction medium (3.08 mM of sodium azide; 0.308 mM β -NADPH, reduced nicotinamide-adenine dinucleotide phosphate; 1.54 U ml^{-1} glutathione reductase and 3.08 mM reduced glutathione in 0.1 M sodium phosphate buffer, pH 7.0). After two minutes, we added 60 μL of 1.5 mM H_2O_2 . The activity was expressed as $\text{nmol min}^{-1}.\text{mg}$ of protein⁻¹.

Glutathione reduced (GSH)

The concentration of GSH was determined by the Sedlak & Lindsay method (1968). A volume of 50 μL of supernatant (after protein precipitation by 50% trichloroacetic acid and centrifugation at 10,000 \times g for 10 min at 4°C) and 230 μL of TRIS (0.4 M, pH 8.9) were placed in a microplate, followed by addition of 20 μL of 2.5 mM DTNB in 25% methanol. Absorbance was determined at 415 nm and we calculated GSH concentration by comparison with the standard curve for GSH and expressed as $\mu\text{g mg}^{-1}$ of protein.

Ethoxyresorufin-O-deethylase (EROD)

The EROD activity was determined according to Burke & Mayer (1974), with spectrofluorimetric determination of resorufin resulting from the metabolism of the 7-ethoxyresorufin by EROD. A volume of 50 μL of sample and 200 μL of reaction solution (2.6 μM 7-ethoxy-resorufin, 0.1 M TRIS, 0.1 M NaCl, pH 7.5) were kept in a microplate and incubated for 5 min. After incubation, we added 10 μL of NADPH (2.6 mM). The fluorimeter measurement was at a wavelength of 530 nm (excitation) and 590 nm (emission) for 10 min at 27°C and the activity expressed as $\text{pmol min}^{-1}.\text{mg}$ of protein⁻¹.

Glutathione S-transferase (GST)

The GST activity was determined based on the procedure described by Keen *et al.* (1976). The reaction is due to the conjugation reaction of the 1-chloro-2,4-dinitrobenzene (CDNB) substrate with reduced glutathione (GSH), catalyzed by GST, forming a thioether. The supernatant (20 μL) was placed in microplate, immediately followed by 180 μL of reaction medium (3 mM GSH, 3 mM CDNB, 0.1 M potassium phosphate buffer, pH 6.5). The absorbance increase was measured at 340 nm and the activity expressed as $\text{nmol min}^{-1}.\text{mg}$ of protein⁻¹.

Total protein quantification

The results from biochemical analyses were normalized to sample protein concentration through the Bradford method (1976). The calibration curve was obtained with bovine serum albumin as the standard.

We carried out the biochemical analyses on a BioTek ELx800 Absorbance Microplate Reader (BioTek Instruments, Inc.).

Statistical analysis

For statistical analysis, we applied the normality test Kolmogorov-Smirnov. For genetic data with no normal distribution, we used the non-parametric test Kruskal-Wallis, and then we compared treatments by Student-Newman-Keuls test. For biochemical assays, ANOVA One-way (Post-test: Test-t LSD) was applied to compare groups. A significance level of $p < 0.05$ was set for all analysis. We performed a multivariate analysis to determine the principal components explaining the data variation.

RESULTS

The titanium dioxide powder was composed of 100% anatase, with a specific surface area (SSA) of $83.47 \text{ m}^2 \text{ g}^{-1}$ (provided by supplier), 107 nm average particle size, and the particles surface chemistry was 28.42% titanium and 71.58% oxygen. The main physical and chemical properties of the suspensions are in Table 1.

We combined a set of biochemical biomarkers to assess the earliest cellular defense mechanisms triggered after chemicals

Table 1: Physical-chemical properties of the nano-TiO₂ suspensions assessed by Zetasizer®.

| Stock solution (mg mL ⁻¹) | Size (d.nm/intensity) | Zeta potential (mV) | Polydispersion stability (%) |
|---------------------------------------|-----------------------|---------------------|------------------------------|
| 0.01 | 338 | -2.99 mV | 91.10 |
| 0.1 | 137.5 | -21.2 mV | 76.48 |
| 1 | 1782 and 5105 | -4.92 mV | 57.27 |

exposure. In the groups treated with metals, Pb and Al, there was a general increase in the activity of all biochemical enzymes levels. The only exceptions were EROD under Pb exposure, and GST to Al.

In relation to the nanoparticles, the lowest concentration (NP1) reduced the activity of SOD and GST, and NP2 reduced SOD and GSH concentration. The NP3 dose alone did not change any of the biomarkers.

In co-exposure treatments of nanoparticles and metal, both GSH and GST decreased in NP1+Al and NP3+Al treatments. In general, we noticed attenuation in the differences of treated

Table 2. Biochemical endpoints in liver of *Hoplias intermedius* after exposure to titanium dioxide nanoparticles-nanoTiO₂ and metals co-exposure.

| | SOD | CAT | GPx | GSH | EROD | GST |
|---------------|-----------------------|---------------------|-------------|----------------------|---------------------|-----------------------|
| NC | 201.8±35.4 | 35.3±4.7 | 59.4±9.7 | 18.7±4.9 | 0.6±0.1 | 288.3±38.1 |
| Pb | 263.7±6.5 * b | 43.2±6.2 * | 71.6±16.3 * | 35.2±10.3 * b | 1.0±0.9 ab | 331.4±33.2 * b |
| Pb+NP1 | 266.6±33.5 * b | 38.7±7.0 | 75.2±9.1 | 29.2±6.5 * b | 1.4±0.4 * b | 362.1±47.7 b |
| NP1 | 163.3±31.6 * a | 38.5±6.3 | 71.9±9.9 | 15.2±2.9 a | 0.7±0.2 a | 167.9±30.3 * a |
| Pb | 263.7±6.5 * b | 43.2±6.2 * | 71.6±16.3 * | 35.2±10.3 * b | 1.0±0.9 | 331.4±33.2 * b |
| Pb+NP2 | 184.2±22.6 a | 47.4±8.0 * | 69.0±13.4 | 24.9±5.2 b | 0.9±0.4 | 355.9±87.2 b |
| NP2 | 162.4±39.4 * a | 42±10.8 | 65.9±23.1 | 11.3±1.1 * a | 0.7±0.4 | 222.2±92.2 a |
| Pb | 263.7±6.5 * b | 43.2±6.2 * | 71.6±16.3 * | 35.2±10.3 * b | 1.0±0.9 | 331.4±33.2 * |
| Pb+NP3 | 186.6±27.3 a | 39.4±7.5 | 67.5±21.3 | 23.3±9.7 a | 1.1±0.3 * | 303.7±77.4 |
| NP3 | 203.4±28.6 a | 38.7±8.9 | 70.9±9.5 | 16.5±3.4 a | 1.0±0.4 | 315.1±65.1 |
| Al | 252.6±52.1 * b | 40.4±6.1 * | 72.4±8.9 * | 25.9±6.9 * b | 2.8±1.7 * b | 289±27.5 b |
| Al+NP1 | 177.8±27.9 a | 35.2±5.7 | 76.8±10.4 * | 13.4±2.8 * a | 3.1±1.6 * b | 173.8±18.7 * a |
| NP1 | 163.3±31.6 * a | 38.5±6.3 | 71.9±9.9 | 15.2±2.9 a | 0.7±0.2 a | 167.9±30.3 * a |
| Al | 252.6±52.1 * b | 40.4±6.1 * | 72.4±8.9 * | 25.9±6.9 * b | 2.8±1.7 * b | 289±27.5 |
| Al+NP2 | 232.4±37.5 b | 34.2±5.7 | 64.9±7.1 | 17.2±3.9 b | 4.1±3.7 * b | 285.8±99.1 |
| NP2 | 162.4±39.4 * a | 42±10.8 | 65.9±23.1 | 11.3±1.1 * a | 0.7±0.4 a | 222.2±92.2 |
| Al | 252.6±52.1 * b | 40.4±6.1 * b | 72.4±8.9 * | 25.9±6.9 * b | 2.8±1.7 * b | 289±27.5 b |
| Al+NP3 | 188.5±51.3 a | 34.3±5.7 a | 66.6±9.3 | 14.6±3.5 * a | 1.3±0.3 * ab | 174.2±46.5 * a |
| NP3 | 203.4±28.6 a | 38.7±8.9 ab | 70.9±9.5 | 16.5±3.4 a | 1.0±0.4 a | 315.1±65.1 b |

The biomarkers values of the activity or concentration are expressed as mean ± standard error. NC: negative control; Pb: 21 µg g⁻¹; Al: 50 µg g⁻¹; NP1: 0.1 µg g⁻¹; NP2: 1.0 µg g⁻¹; NP3: 10 µg g⁻¹ of nano-TiO₂. * indicates significant difference with negative control ($p < 0.05$). We used different letters to indicate synergic effects among each set of metal and nanoparticle co-exposure, the purpose is to show if the mixture present higher or lower toxicity than the chemicals apart ($p < 0.05$). SOD (U mg⁻¹.protein⁻¹), CAT (µmol min⁻¹.mg of protein⁻¹), GPx and GST (nmol min⁻¹.mg of protein⁻¹), GSH (µg mg⁻¹ of protein⁻¹) and EROD (pmol min⁻¹.mg of protein⁻¹).

groups when compared to control. Usually, animals treated with mixed metal and nano-TiO₂ did not differ from the negative control, in contrast with the group exposed only to metals. Metals do have a stronger ability to interfere in the biochemical enzymes. This can be clearly seen in the case of SOD, and predominantly, in the CAT and GPx (Table 2).

EROD activity did not increase with Pb alone, but when it was associated with nano-TiO₂ in the lowest and highest concentration, the enzyme activity increased without a dose-specific pattern to nano-TiO₂, but co-exposure caused an imbalance of activity of various biochemical biomarkers.

Nanoparticles by themselves did not induce DNA damage, whilst both metals did (Fig.2).

Principal components analysis (PCA) is a qualitative demonstration of the combined data and it also shows data variation. For the first dataset (NC, NPs, Pb, and NPs+Pb) the two principal components contain 75.03% of the variation from the seven original variables (Fig. 3). For the analysis including the aluminium and nanoparticles (NC, NPs, Al and NPs+Al), the two principal components contain 61.58% of the variation. In addition, the comet assay and the EROD activity were the most representative biomarkers in the study, as they explain the main components of the PCA (Fig. 4).

DISCUSSION

We prepared three different suspensions, in three concentrations, to ensure that the volume injected in each animal were the same. Besides the agglomeration of the particles, which was elevated in the most concentrated suspension, all of them had an unstable Zeta potential ($+30 \text{ mV} > \zeta > -30 \text{ mV}$). The magnitude of the Zeta potential indicates the degree of electrostatic repulsion between adjacent charged particles in a dispersion. If all the particles in suspension have low zeta potential values then there is no force to prevent the particles coming together and flocculating. Particles with zeta potentials more positive than +30mV or more negative than -30mV are normally considered stable (Malvern Instruments, 2005). The Zeta potential is a key indicator of the stability of colloidal dispersions that also presented a heterogeneous size pattern (polydispersion percentage higher than 20%), so not all the particles had the same size, even though they were around a core peak.

The wide variability in the nanoparticle research findings may be due to different chemical characteristics of nano-TiO₂ as well as experimental design. It is why comparisons between physical-chemical characteristics and *in vivo* endpoints are

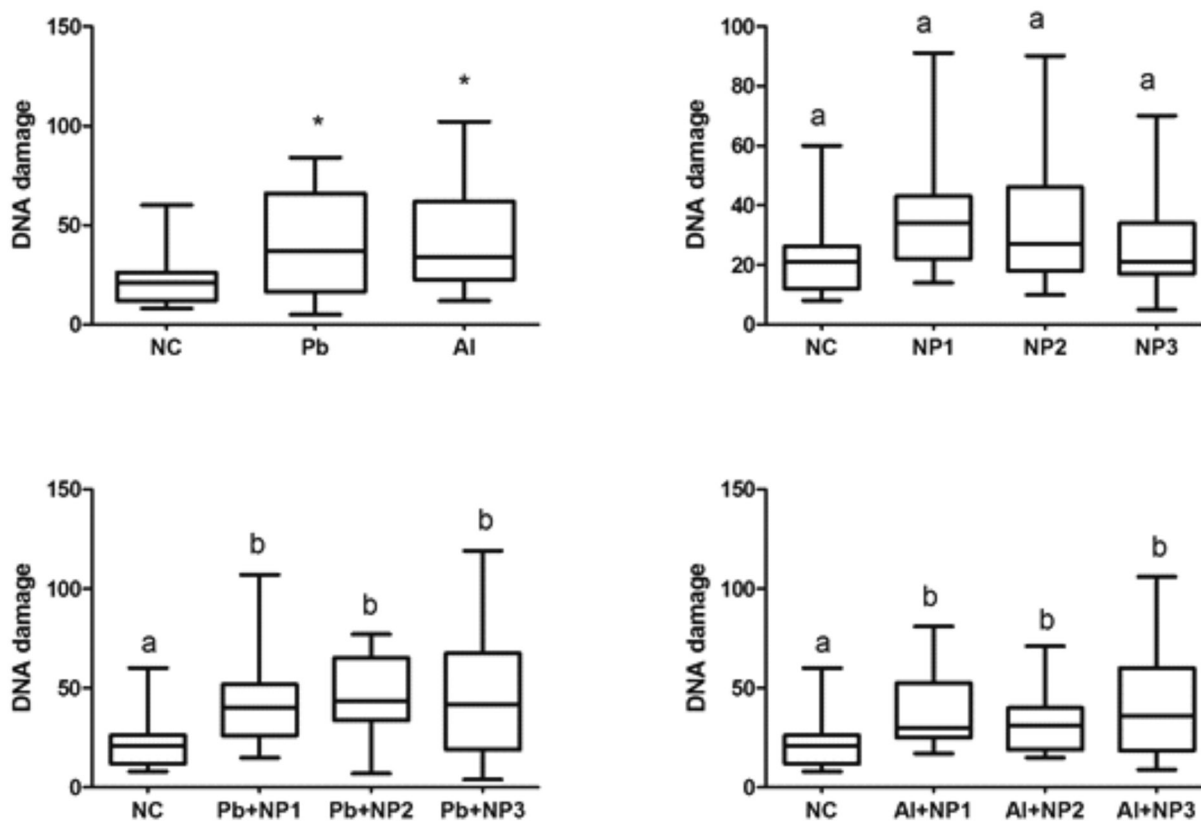


Figure 2: DNA damage assessed by comet assay in liver cells after acute exposure (96 h) of Titanium Dioxide Nanoparticles (nano-TiO₂) at the doses 0.1; 1 and 10 $\mu\text{g g}^{-1}$; (NP1, NP2, and NP3, respectively) and co-exposure with lead (Pb 21 $\mu\text{g g}^{-1}$) and aluminium (50 $\mu\text{g g}^{-1}$). The box and whisker plots show the median and the first and third quartiles. * represent statistically difference with the negative control (NC) and different letters indicate differences among treatments. Level of significance $p < 0.05$.

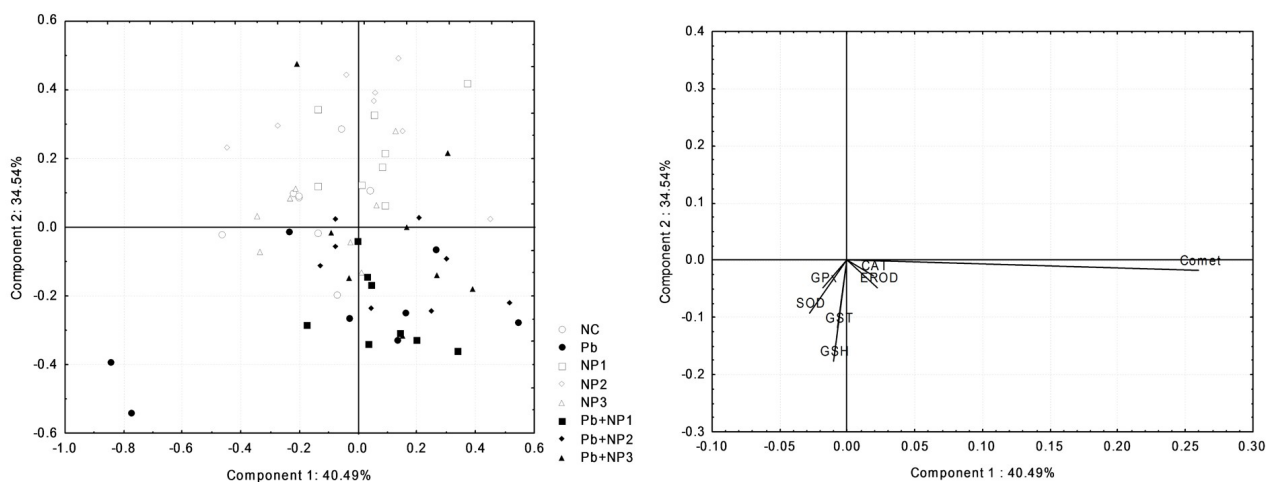


Figure 3: Principal component analysis (PCA) of the dataset composed for negative control (NC), lead ($\text{Pb } 21 \mu\text{g g}^{-1}$), nano- TiO_2 $0.1 \mu\text{g g}^{-1}$ (NP1), nano- TiO_2 $1 \mu\text{g g}^{-1}$ (NP2), nano- TiO_2 $10 \mu\text{g g}^{-1}$ (NP3), and NPs+Pb.

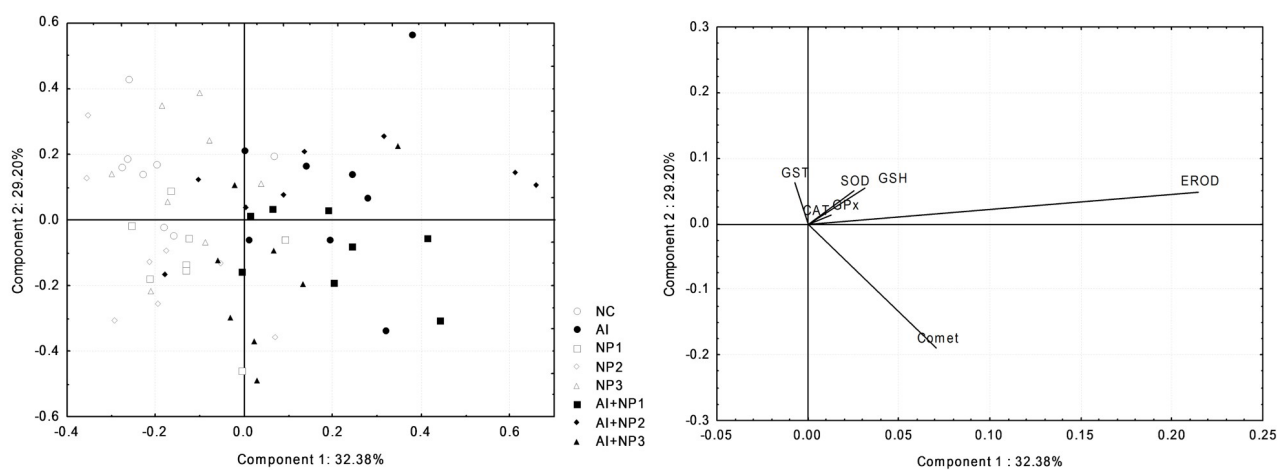


Figure 4: Principal component analysis (PCA) of the dataset composed for negative control (NC), aluminium ($50 \mu\text{g g}^{-1}$), nano- TiO_2 $0.1 \mu\text{g g}^{-1}$ (NP1), nano- TiO_2 $1 \mu\text{g g}^{-1}$ (NP2), nano- TiO_2 10

important to assess the biological responses. The elevated average size of the agglomerated nanoparticles is due to multiple primary particles and aggregates joined together by Van der Waal forces (Fig. 1). These particles can break off when the micro environment conditions change, for example, after the entrance of an organism. Small particles may be released from large clusters that could not enter cells easily, eventually causing undesirable effects (US FDA, 2014).

Available scientific information does not establish a uniform upper boundary above 100 nm , where novel properties and phenomena similar to those seen in materials with dimensions in the nanoscale range cease. For this reason, Food and Drug Administration-FDA finds it reasonable to consider evaluation of materials with dimensions up to $1,000 \text{ nm}$, as a mean to screen material for further examination and to determine whether these materials exhibit characteristics related to the application of nanotechnology (US FDA, 2014).

However, it is important to note it is a somewhat arbitrary size cut off from the ecotoxicity bias. It might be prudent

to consider aggregates of NPs that can be a few hundred nanometers wide (Federici *et al.*, 2007), or with a distribution of particles around the nanoscale, but having some primary particles larger than 100 nm (Handy *et al.*, 2008).

Regarding the particle size, all the NPs suspensions prepared had an average size higher than 100 nm , while the most concentrated had two peaks, both higher than $1,000 \text{ nm}$. Adverse effects at sublethal concentrations is extremely important in environmental assessment, since it may generate a cascade effect with consequences at the individual level, community, up to the ecosystem (Wu *et al.*, 2016).

Biochemical biomarkers offer the advantage of detection potentially toxic exposure well before adverse effects occur. In this study, several endpoints were included to understand the toxicological aspects of nano- TiO_2 and their interaction with metal compounds. Metals are substances with known toxic potential and because some biomarkers are highly sensitive, such change was expected. In relation to the nanoparticles, the biochemical endpoints were more affected

by the low concentrations (NP1 and NP2), possibly because the size of some particles in the suspension was tiny. The highest concentration of nanoparticle did not interfere in the biochemical enzymes; it must be due to the size of the particles, which were predominantly greater at this concentration than at any other (Table 1). This confirms the fact that the aggregate size has a direct influence on biological responses.

Superoxide dismutase (SOD) is an essential metalloenzyme to the antioxidant defense system as it catalyzes the dismutation of the superoxide radical (-O_2) to form hydrogen peroxide (H_2O_2). In addition, both the GST and GSH are important at antioxidant defense, removing oxygen radicals and reactivating intermediates, protecting cells against oxidative damage.

GSH can react with oxidant species well before they interact with macromolecules (Pompella *et al.*, 2003). GSH and GST decreased in some co-exposure treatments, and this can be explained by the use of GSH as a cofactor of GST in cellular dynamics, as GST catalyzes the conjugation of glutathione with xenobiotics (Jemec *et al.*, 2010), thereby decreasing the compound. Therefore, a non-enzymatic cofactor can lead to a decrease of the enzyme that the cofactor is associated. Thus, the decrease of these biochemical biomarkers after exposure to nano-TiO₂ indicates a possible negative effect on the fish defenses to xenobiotics. Still, the reduction in enzyme activity can occur by binding of NPs or metabolites to these proteins.

Catalase decomposes the hydrogen peroxide, which is more stable than the superoxide anion, but it can also cause cell damage by ROS. Some of the H_2O_2 is produced by the cell through SOD, so when the SOD activity reduces, consequently, the activity of CAT is diminished or kept constant, due to less hydrogen peroxide being available to be decomposed by CAT.

Early contamination biomarkers are important because they respond in subcellular level and at the expense of any imbalance of homeostasis and physiological instability, even by low doses of chemical contamination. In ecotoxicological research, biochemical biomarkers are considered the most promising tool for such purposes, being early indicators for environmental disturbances, with quick response to stress.

The metabolic transformation of chemicals within the organisms is fundamental to change the compound biological activity and, as a result, decrease or increase chemical-cell interaction. Biotransformation includes numerous different enzymatic systems, which act over a variety of substrates. The main set of enzymes related with early biotransformation reactions- phase I- are the flavoprotein monooxygenases and heme proteins. On the other hand, phase II reactions can be used to infer both exposure and effect, since a variety of xenobiotics can modify their activity (Sies, 1999).

In a study where carps (*Cyprinus carpio*) were exposed to 100 and 200 mg L⁻¹ of nano-TiO₂, the treatment caused statistically significant decrease in SOD and CAT, suggesting that the fish exposed to these NPs suffered from oxidative stress (Linhua *et al.*, 2009). Furthermore, Federici *et al.* (2007) measured nano-TiO₂ toxicity to rainbow trout after 14

days of exposure; it caused significant increases in the total glutathione levels in the gills and significant decreases in Na⁺K⁺-ATPase activity. They also related depletion of hepatic glutathione compared to controls, and some hepatocytes showed condensed nuclear bodies, indicating apoptosis.

In this study, nano-TiO₂ did not increase DNA damage, which indicates absence of genotoxicity in the tested doses under our tested conditions although different effects may be observed with different NP doses or exposures. As an example, Vignardi *et al.* (2015) exposed the marine fish *Trachinotus carolinus*, by intraperitoneal injection, to 1.5 and 3.0 µg g⁻¹ nano-TiO₂ and the results indicated genotoxicity and potentially cytotoxicity.

Xiong *et al.* (2011) studied the acute toxicity and oxidative effects of nano-scale titanium dioxide and their bulk counterparts in zebrafish. They have found that although the size distribution of nanoparticles was similar to bulk particles in suspension, the acute toxicity of the nano-TiO₂ to zebrafish was greater than of the bulk material, especially through a great generation of $\cdot\text{OH}$, while bulk particles were essentially non-toxic. Like in this study, the highest dose of NPs tested had in his colloidal suspension particles of large size (>1,000 nm), and probably because of that, the effects were bland. Although not considered as bulk yet, it is remarkable that the increase in particle size has a direct influence on the reduction of toxicity, as the hazard of some metallic NPs might be different to the traditional dissolved forms of metals (Shaw & Handy, 2011).

In the natural environment, there are many different compounds and most of them are present at low concentrations. The considerable raising concerns over toxicity is particularly essential when they are present as components of complex mixtures. Adverse effects would be caused not only by the nanomaterials themselves, but also to the capacity of NPs to modify bioavailability of other toxic pollutants such as heavy metals and toxic organic compounds (Rossi *et al.*, 2014).

There is evidence of “delivery effects” when the metal is present as a co-contaminant with a nanoparticle. This is related to the ability of metals to adsorb to the surface of some negatively charged NPs (Handy *et al.*, 2008). Other studies have shown that the presence of nano-TiO₂ may elevate the absorption of other contaminants in fish. For example, Zhang *et al.* (2007) exposed carps to cadmium for 25 days in the presence of nano-TiO₂ and found that the fish accumulated 146% more Cd compared to fish exposed only to Cd.

In the same way, TiO₂ nanoparticles had a significant adsorption capacity for Arsenic. Sun *et al.* (2007) exposed carp to the metalloid arsenic-As (V) in the presence of nano-TiO₂ and fish accumulated 132% more As than the treatment without nanoparticles. Most importantly, besides the enhanced metal accumulation when associated with TiO₂, the contaminants generally are able to interact and to alter some biomarkers. Rossi *et al.* (2014) also assessed the modulatory effect of nano-TiO₂ on Pb and they have found hepatic and neural effects over the fish *Hoplias malabaricus*.

Reeves *et al.* (2008) evaluated the *in vitro* cytotoxic and genotoxic potential effects of TiO₂ nanoparticles on goldfish

skin cells (GFSk-S1) treated with 1.0, 10 and 100 $\mu\text{g ml}^{-1}$ nano-TiO₂ for only 24 hours. They have found that all doses caused significant increases in oxidative DNA damage. Therefore, for these fish cells, the nanoparticles were in fact genotoxic, but they could not conclude which sort of radical species was responsible for the DNA break effects, although it appears to be likely the $\cdot\text{OH}$. Accordingly, fish cells are generally more susceptible to toxic/oxidative injury than similarly treated mammalian cells.

Vevers & Jha (2008) also confirmed intrinsic genotoxic and cytotoxic potential of TiO₂ ENPs by the induction of DNA strand breaks, including oxidative damage to the DNA, and lysosomal membrane integrity in a metabolically competent fish cell line derived from rainbow trout (*Oncorhynchus mykiss*) gonadal tissue. It corroborates the evidence that fish cell lines can also provide important information on nanoparticles possible adverse effects.

In ecotoxicological studies, it is important to apply general and holistic approaches to evaluate biological responses to contaminants. A battery of biomarkers from different levels of biological organization can adequately identify hazard, and this is because the organisms used in the studies offer several endpoints that depend on their sensitivity, on the mode of action of the tested compounds, and time of exposure (Jemec *et al.*, 2010). A degree of ambiguity or inconclusiveness is inherent in some findings from certain assays as applied to nanosystems, sometimes due to intrinsic challenges associated with the analysis of nanomaterials (Jones & Grainger, 2009).

We used PCA to find patterns in the dataset and provide a concise approach and simple visualization of the data obtained from a large number of variables and measurements. We combined a set of data including the groups exposed to nanoparticles and mixed with each metal (Pb or Al). In the first situation, all biomarkers pointed out to the Pb direction in the data distribution, independently of the TiO₂ nanoparticles. All and every treatment containing Pb tends to cause the grouping of the data. In this general context, the nanoparticles seems not to be greatly relevant considering this overview created by a multivariate analysis, corroborating the weak toxicity of this compound found out during the tests. The fact that the main components were representative for an expressive percentage of variation is interesting because it demonstrated the low variability among the collected data. For the analysis including the aluminium and nanoparticles, again all the biomarkers tend to direct to the treatments including the Al. Then, as expected, the metals disturbed the animals' metabolism, and nano-TiO₂ did not really interfered in the process.

CONCLUSION

Nano-TiO₂ was not genotoxic to the fish *Hoplias intermedius* when evaluated by the comet assay in the experimental conditions, although in co-exposure it was able to change the levels of some important biochemical biomarkers compared to the control or metals exposure.

Interactions among nanoparticles and other contaminants present in complex mixtures may be harmful by depleting the cellular defenses or restrain the detoxification mechanisms from functioning properly, at least in the studied fish.

ACKNOWLEDGMENTS

The authors thank the Pisciculture and Hydrobiology Station of Furnas for the donation of the specimens; the professor Dr. Carmen Lúcia Voigt from State University of Ponta Grossa for the assistance with nanoparticle analysis; CNPq (Brazilian Agency for Science and Technology) and Fundação Araucária for the financial support.

REFERENCES

- AEBI, H. 1984. Catalase *in vitro*. *Methods Enzymol*, 105: 121-126.
- BRADFORD, M.M. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal Biochem*, 72: 248-254.
- BURKE, M.D. & MAYER, R.T. 1974. Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab Dispos*, 2(6): 583-588.
- CLEMENTE, Z., CASTRO, V.L., JONSSON, C.M. & FRACETO, L.F. 2012. Ecotoxicology of Nano-TiO₂- An Evaluation of its Toxicity to Organisms of Aquatic Ecosystems. *Int. J. Environ Res*, 6(1): 33-50.
- COLLINS, A.R., DOBSON, V.L., DUŠINSKÁ, M., KENNEDY, G. & ŠTĚTINA, R. 1997. The comet assay: What can it really tell us? *Mutat Res*, 375: 183-193. [https://doi.org/10.1016/S0027-5107\(97\)00013-4](https://doi.org/10.1016/S0027-5107(97)00013-4)
- COSTA, P.M. 2011. Avaliação do efeito tóxico de sulfato de alumínio e sulfato de cobre em bioensaio de contaminação subcrônica via trófica no bioindicador *Rhamdia quelen* (Siluriforme). PhD Thesis. Universidade Federal do Paraná, Curitiba, 117p.
- CRANE, M., HANDY, R.D., GARROD, J. & OWEN, R. 2008. Ecotoxicity test methods and environmental hazard assessment for engineered nanoparticles. *Ecotoxicology*, 17: 421-437. <http://dx.doi.org/10.1007/s10646-008-0215-z>
- FEDERICI, G., SHAW, B.J. & HANDY, R.D. 2007. Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): Gill injury, oxidative stress, and other physiological effects. *Aquat Toxicol*, 84: 415-430. <http://dx.doi.org/10.1016/j.aquatox.2007.07.009>
- FERRARO, M.V.M., FENOCCHIO, A.S., MANTOVANI, M.S., RIBEIRO, C.O. & CESTARI, M.M. 2004. Mutagenic effects of tributyltin and inorganic lead (Pb II) on the fish *H. malabaricus* as evaluated using the comet assay and the piscine micronucleus and chromosome aberration tests. *Genet Mol Biol*, 27(1): 103-2017. <http://dx.doi.org/10.1590/S1415-47572004000100017>
- GAMELIN, F.X., BAQUET, G., BERTHOIN, S., THEVENET, D., NOURRY, C., NOTTIN, S. & BOSQUET, L. 2009. Effect of high intensity intermittent training on heart rate variability in prepubescent children. *Eur J Appl Physiol*, 105: 731-738. <http://dx.doi.org/10.1007/s00421-008-0955-8>
- GAO, R., YUAN, Z. & ZHAO, Z. 1998. Mechanism of pyrogallol autoxidation and determination of superoxide dismutase enzyme activity. *Bioelectrochem Bioenerg*, 45: 41-45. [https://doi.org/10.1016/S0302-4598\(98\)00072-5](https://doi.org/10.1016/S0302-4598(98)00072-5)
- GONTIJO, A.M., BARRETO, R.E., SPEIT, G., VALENZUELA REYES, V.A., VOLPATO, G.L. & FAVERO SALVADORI, D.M. 2003. Anesthesia of fish with benzocaine does not interfere with comet assay results. *Mutat Res*, 534: 165-72. [https://doi.org/10.1016/S1383-5718\(02\)00276-0](https://doi.org/10.1016/S1383-5718(02)00276-0)

- GOKSOYR, A. 1995. Use of cytochrome P450 1A (CYP1A) in fish as a biomarker of aquatic pollution. *Arch Toxicol*, 17: 80-95.
- GROSELL, M.H., HOGSTRAND, C. & WOOD, C.M. 1998. Renal Cu and Na excretion and hepatic Cu metabolism in both Cu acclimated and non acclimated rainbow trout (*Oncorhynchus mykiss*). *Aquat Toxicol*, 40: 275-91. [https://doi.org/10.1016/S0166-445X\(97\)00026-X](https://doi.org/10.1016/S0166-445X(97)00026-X)
- HANDY, R.D., HENRY, T.B., SCOWN, T.M., JOHNSTON, B.D. & TYLER, C.R. 2008. Manufactured nanoparticles: their uptake and effects on fish — a mechanistic analysis. *Ecotoxicology*, 17: 396-409. <https://doi.org/10.1007/s10646-008-0205-1>
- JEMEC, A., DROBNE, D., TISLER, T. & SEPCIC, K. 2010. Biochemical biomarkers in environmental studies- lessons learnt from enzymes catalase, glutathione S-transferase and cholinesterase in two crustacean species. *Environ Sci Pollut Res Int*, 17 (3): 571-581. <https://doi.org/10.1007/s11356-009-0112-x>
- JONES, C.F. & GRAINGER, D.W. 2009. *In vitro* assessments of nanomaterial toxicity. *Adv Drug Deliv Rev*, 61: 438-456. <https://doi.org/10.1016/j.addr.2009.03.005>
- JOVANOVIC, B. & PALIC, D. 2012. Immunotoxicology of non-functionalized engineered nanoparticles in aquatic organisms with special emphasis on fish- Review of current knowledge, gap identification, and call for further research. *Aquat Toxicol*, 118: 141-151. <https://doi.org/10.1016/j.aquatox.2012.04.005>
- KAIN, J., KARLSSON, H.L. & MÖLLER, L. 2012. DNA damage induced by micro- and nanoparticles- interaction with FPG influences the detection of DNA oxidation in the comet assay. *Mutagenesis*, 27 (4): 491-500. <https://doi.org/10.1093/mutage/ges010>
- KAUR, A. & GRUPTA, U. 2009. A review on applications of nanoparticles for the pre concentration of environmental pollutants. *J Mater Chem*, 19: 8279-8289.
- KEEN, J.H., HABIG, W.H. & JAKOBY, W.B. 1976. Mechanism for several activities of the glutathione S-transferase. *J Biol Chem*, 251: 6183-6188.
- KELLER, A.A., MCFERRAN, S., LAZAREVA, A. & SUH, S. 2013. Global life cycle releases of engineered nanomaterials. *J Nanopart Res*, 15: 1692. <https://doi.org/10.1007/s11051-013-1692-4>
- KLINGELFUS, T., COSTA, P.M., SCHERER, M. & CESTARI, M.M. 2015. DNA damage in the kidney tissue cells of the fish *Rhamdia quelen* after trophic contamination with aluminium sulfate. *Genet Mol Biol*, 38 (4): 499-506. <https://doi.org/10.1590/S1415-475738420140327>.
- KUMAR, A., PANDEY, A.K., SINGH, S.S., SHANKER, R. & DHAWAN, A. 2011. Cellular uptake and mutagenic potential of metal oxide nanoparticles in bacterial cells. *Chemosphere*, 83: 1124-1132. <https://doi.org/10.1016/j.chemosphere.2011.01.025>
- LAVICOLI, L., LESO, V., FONTANA, L. & BERGAMASCHI, A. 2011. Toxicological effects of titanium dioxide nanoparticles: a review of in vitro mammalian studies. *Eur Rev Med Pharmacol Sci*, 15 (5): 481-508.
- LINHUA, H., WANG, Z. & XING, B. 2009. Effect of sub-acute exposure to TiO₂ nanoparticles on oxidative stress and histopathological changes in Juvenile Carp (*Cyprinus carpio*). *J Environ Sci*, 21 (10): 1459-1466. [https://doi.org/10.1016/S1001-0742\(08\)62440-7](https://doi.org/10.1016/S1001-0742(08)62440-7)
- MALVERN INSTRUMENTS Ltd. 2005. Zetasizer Nano Series- User manual. Nano 317, issue 2.2, Worcestershire, United Kingdom.
- NAKAGAWA, Y., WAKURI, S., SAKAMOTO, K. & TANAKA, N. 1997. The photogenotoxicity of titanium dioxide particles. *Mutat Res*, 394: 125-132. [https://doi.org/10.1016/S1383-5718\(97\)00126-5](https://doi.org/10.1016/S1383-5718(97)00126-5)
- PAGLIA, D.E. & VALENTINE, W.N. 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med*, 70 (1): 158-169.
- POMPELLA, A., VISVIKIS, A., PAOLICCHI, A., TATA, V. & CASINI, F. 2003. The changing faces of glutathione, a cellular protagonist. *Biochem Pharmacol*, 66: 1499-1503. [https://doi.org/10.1016/S0006-2952\(03\)00504-5](https://doi.org/10.1016/S0006-2952(03)00504-5)
- RAMSDORF, W.A., FERRARO, M.V.M., OLIVEIRA-RIBEIRO, C.A., COSTA, J.R.M. & CESTARI, M.M. 2009. Genotoxic evaluation of different doses of inorganic lead (PbII) in *Hoplias malabaricus*. *Environ Monit Assess*, 158: 77-85. <https://doi.org/10.1007/s10661-008-0566-1>
- REEVES, J.F., DAVIES, S.J., DODD, N.J.F. & JHA, A.N. 2008. Hydroxyl radicals ($\cdot\text{OH}$) are associated with titanium dioxide (TiO₂) nanoparticle-induced cytotoxicity and oxidative DNA damage in fish cells. *Mutat Res*, 640 : 113-122. <https://doi.org/10.1016/j.mrfmmm.2007.12.010>
- ROSENFELDT, R.R., SEITZ, F., ZUBROD, J.P., FECKLER, A., MERKEL, T., LÜDERWALD, S., BUNDSCHUH, R., SCHULZ, R. & BUNDSCHUH, M. 2015. Does the presence of titanium dioxide nanoparticles reduce copper toxicity? A factorial approach with the benthic amphipod *Gammarus fossarum*. *Aquat Toxicol*, 165: 154-159. <https://doi.org/10.1016/j.aquatox.2015.05.011>
- ROSSI, S.C., MELA, M., BOSCHEN, S.L., CUNHA, C., FILIPAK NETO, F., OLIVEIRA RIBEIRO, C.A., NEVES, A.P.P. & SILVA DE ASSIS, H.C. 2014. Modulatory effect of nano TiO₂ on Pb in *Hoplias malabaricus* trophically exposed. *Environ Toxicol Pharmacol*, 38: 71-78. <https://doi.org/10.1016/j.etap.2014.05.013>
- RYBAK M., KOŁODZIEJCZYK A., JONIAK T., RATAJCZAK I. & GABKA M. 2017. Bioaccumulation and toxicity studies of macroalgae (Charophyceae) treated with aluminium : Experimental studies in the context of lake restoration. *Ecotoxicol Environ Saf*, 145 : 359-366. <https://doi.org/10.1016/j.ecoenv.2017.07.056>
- SAGHAZADEH A. & REZAEIN. 2017. Systematic review and meta-analysis links autism and toxic metals and highlights the impact of country development status : Higher blood and erythrocyte levels for mercury and lead, and higher hair antimony, cadmium, lead, and mercury. *Prog Neuropsychopharmacol Biol Psychiatry*, 79 : 340-368. <https://doi.org/10.1016/j.pnpbp.2017.07.011>
- SCOWN, T.M., AERLE, R., JOHNSTON, B.D., CUMBERLAND, S., LEAD, J.R., OWEN, R. & TYLER, C.R. 2009. High doses of intravenously administered titanium dioxide nanoparticles accumulate in the kidney of rainbow trout but with no observable impairment of renal function. *Toxicol Sci*, 109(2): 372-380. <https://doi.org/10.1093/toxsci/kfp064>
- SEDLAK, J. & LINDSAY, R.H. 1968. Estimation of total protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem*, 21(1): 192-205.
- SHAW, B.J. & HANDY, R.D. 2011. Physiological effects of nanoparticles on fish: A comparison of nanometals versus metal ions. *Environ Int*, 37: 1083-1097. <https://doi.org/10.1016/j.envint.2011.03.009>
- SIES, H. 1999. Glutathione and its role in cellular functions. *Free Radic Biol Med*, 27 (9/10): 916-921.
- SINGH, N.P., McCOY, M.T., TICE, R.R. & SCHNEIDER, E.L.A. 1988. Simple technique for quantification of low levels of DNA damage in individual cells. *Exp Cell Res*, 175: 184-191.
- SUN, H., ZHANG, X., NIU, Q., CHEN, Y. & CRITTENDEN, J.C. 2007. Enhanced accumulation of arsenate in carp in the presence of titanium dioxide nanoparticles. *Water Air Soil Pollut*, 178: 245-54. <https://doi.org/10.1007/s11270-006-9194-y>

- SUN, T.Y., GOTTSCHALK, F., HUNGERBÜHLER, K. & NOWACK, B. 2014. Comprehensive probabilistic modeling of environmental emissions of engineered nanomaterials. *Environ Pollut*, 185: 69-76. <https://doi.org/10.1016/j.envpol.2013.10.004>
- TAN, C. & WANG, W.X. 2017. Influences of TiO₂ nanoparticles on dietary metal uptake in *Daphnia magna*. *Environ Pollut*, 231: 311-318. <https://doi.org/10.1016/j.envpol.2017.08.024>
- TAVARES, A.M., LOURO, H., ANTUNES, S., QUARRÉ, S., SIMAR, S., TEMMERMAN, P.J., VERLEYSEN, E., MAST, J., JENSEN, K.A., NORPPA, H., NESSLANY, F. & SILVA, M.J. 2014. Genotoxicity evaluation of nanosized titanium dioxide, synthetic amorphous silica and multi-walled carbon nanotubes in human lymphocytes. *Toxicol In Vitro*, 28: 60-69. <http://doi.org/10.1016/j.tiv.2013.06.009>
- US FDA (Food and Drug Administration). 2014. Guidance for Industry- Considering Whether an FDA-Regulated Product Involves the Application of Nanotechnology. <http://www.fda.gov/RegulatoryInformation/Guidances/ucm257698.htm>.
- VANCE, M.E., KUIKEN, T., VEJERANO, E.P., MCGINNIS, S.P., HOHELLA, M.F., REJESKI, D. & HULL, M.S. 2015. Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. *Beilstein J Nanotechnol*, 6: 1769-1780. <http://dx.doi.org/10.3762/bjnano.6.181>
- VIGNARDI, C.P., HASUE, F.M., SARTÓRIO, P.V., CARDOSO, C.M., MACHADO, A.S.D., PASSOS, M.J.A.C.R., SANTOS, T.C.A., NUCCI, J.M., HEWER, T.L.R., WATANABE, I.S., GOMES, V. & PHAN, N.V. 2015. Genotoxicity, potential cytotoxicity and cell uptake of titanium dioxide nanoparticles in the marine fish *Trachinotus carolinus* (Linnaeus, 1766). *Aquat Toxicol*, 158: 218-229. <http://doi.org/10.1016/j.aquatox.2014.11.008>
- WANG, G., JIN, W., QASIM, A.M., GAO, A., PENG, X., LI, W., FENG, H. & CHU, P.K. 2017. Antibacterial effects of titanium embedded with silver nanoparticles based on electron-transfer-induced reactive oxygen species. *Biomaterials*, 124: 25-34. <https://doi.org/10.1016/j.biomaterials.2017.01.028>
- WU, B., LI, X., SONG, J., HU, L. & SHI, X. 2016. Impact of extreme metal contamination at the supra-individual level in a contaminated bay ecosystem. *Sci Total Environ*, 102-9: 557-558. <http://doi.org/10.1016/j.scitotenv.2016.03.047>
- XIONG, D., FANG, T., YU, L., SIMA, X. & ZHU, W. 2011. Effects of nano-scale TiO₂, ZnO and their bulk counterparts on zebrafish: Acute toxicity, oxidative stress and oxidative damage. *Sci Total Environ*, 409: 1444-1452. <http://doi.org/10.1016/j.scitotenv.2011.01.015>
- ZHANG, X., SUN, H., ZHANG, Z., NIU, Q., CHEN, Y. & CRITTENDEN, J.C. 2007. Enhanced bioaccumulation of cadmium in carp in the presence of titanium dioxide nanoparticles. *Chemosphere*, 67: 160-166. <http://doi.org/10.1016/j.chemosphere.2006.09.003>